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Seven *Dictyostelium discoideum* phosphodiesterases degrade three pools of cAMP and cGMP

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The *Dictyostelium discoideum* genome uncovers seven cyclic nucleotide PDEs (phosphodiesterases), of which six have been characterized previously and the seventh is characterized in the present paper. Three enzymes belong to the ubiquitous class I PDEs, common in all eukaryotes, whereas four enzymes belong to the rare class II PDEs that are present in bacteria and lower eukaryotes. Since all *D. discoideum* PDEs are now characterized we have calculated the contribution of each enzyme in the degradation of the three important pools of cyclic nucleotides: (i) extracellular cAMP that induces chemotaxis during aggregation and differentiation in slugs; (ii) intracellular cAMP that mediates development; and (iii) intracellular cGMP that mediates chemotaxis. It appears that each cyclic nucleotide pool is degraded by a combination of enzymes that have different affinities, allowing a broad range of substrate concentrations to be degraded with first-order kinetics. Extracellular cAMP is degraded predominantly by the class II high-affinity enzyme DdPDE1 and its close homologue DdPDE7, and in the multicellular stage also by the low-affinity transmembrane class I enzyme DdPDE4. Intracellular cAMP is degraded by the DdPDE2, a class I enzyme regulated by histidine kinase/phospho-relay, and by the cAMP-/cGMP-stimulated class II DdPDE6. Finally, basal intracellular cGMP is degraded predominantly by the high-affinity class I DdPDE3, while the elevated cGMP levels that arise after receptor stimulation are degraded predominantly by a cGMP-stimulated cGMP-specific class II DdPDE5. The analysis shows that the combination of enzymes is tuned to keep the concentration and lifetime of the substrate within a functional range.

Key words: cAMP, cGMP, chemotaxis, development, *Dictyostelium discoideum*, phosphodiesterase.

INTRODUCTION

The cyclic nucleotides cAMP and cGMP play a central role in diverse signal transduction processes in *Dictyostelium discoideum*. cAMP mediates chemotaxis during cell aggregation, and controls gene expression during development. cGMP regulates cytoskeletal organization affecting shape, stability and motility of single cells. The intracellular concentration of cAMP and cGMP is determined by the combined action of production and removal. Production depends on the enzymatic activity of the adenylyl and guanylyl cyclases to form cAMP and cGMP respectively. Removal of intracellular cAMP or cGMP depends on the activity of PDEs (phosphodiesterases) that hydrolyse cAMP and cGMP, and on the ability of *D. discoideum* cells to secrete cAMP. This extrusion mechanism is pivotal in the formation of extracellular cAMP waves, which is the chemotactic signal for cell-directed movement during aggregation, mound formation and slug migration.

In *D. discoideum* cAMP is produced by three adenylyl cyclases (see [1]): ACA is a transmembrane enzyme that is regulated by G-protein-coupled receptors [2], ACB, encoded by the *acrA* gene [3], is a soluble adenylyl cyclase with high basal activity [4], whereas ACG is an osmosensitive adenylyl cyclase [1]. cGMP synthesis is mediated by two guanylyl cyclases, the transmembrane GCA and the soluble sGC that are both regulated by G-protein-coupled receptor pathways (see [5]). For the degradation of cAMP and cGMP, six different PDEs have been reported and characterized in *D. discoideum*. These PDEs represent two classes that exhibit distinct differences in the amino acid sequence of the putative catalytic domains, namely class I, which is ubiquitous in mammals, and class II that predominantly occurs in bacteria and lower eukaryotes (see Figure 1).

We will follow the nomenclature of *D. discoideum* PDEs that has been proposed by Bosgraaf et al. [6] and will give original or alternative names on first mentioning. DdPDE1 (PdsA) is a class II dual-specificity PDE that degrades extracellular cAMP and cGMP. The enzyme can be secreted in the medium, or exposed on the cell surface. Cells without DdPDE1 (*pdsA*-null strain UK7) reveal that DdPDE1 is the main PDE that degrades extracellular cAMP during cell aggregation and is thereby essential for shaping cAMP waves. UK7 cells fail to aggregate, but can be rescued by adding exogenous PDE activity [7–17]. DdPDE2 (RegA) encodes a cAMP-specific PDE localized in the cytosol of the cell where it degrades intracellular cAMP. The protein harbours a class I PDE catalytic domain and a response regulator domain, which is phosphorylated and activated by histidine kinases via the phospho-relay protein RdeA [18]. The DdPDE2 knockout cell strain, *regA*$^-$, develops into very small aggregates and shows defects in spore formation. Additionally DdPDE2 has been implicated in suppression of pseudopodium formation during chemotaxis [4,19–26]. DdPDE3 is a cGMP-specific class I PDE that consists of a catalytic domain without regulatory domains, and is constitutively active. The *ddpde3*$^-$ null cell strain shows a moderate phenotype with increased basal cGMP levels, but only a small effect on cAMP-stimulated cGMP levels compared with wild-type cells [27]. DdPDE4 is a class I cAMP-specific enzyme with two transmembrane segments and an extracellular catalytic domain. The protein is expressed mainly in the multicellular stage where it is involved in the degradation of extracellular cAMP. The *ddpde4*$^-$ null cell strain shows normal cell aggregation, but is

Abbreviations used: cNB, cyclic nucleotide-binding; DTT, dithiothreitol; GFP, green fluorescent protein; GPI, glycosylphosphatidylinositol; IBMX, 3-isobutyl-1-methylxanthine; PDE, phosphodiesterase.

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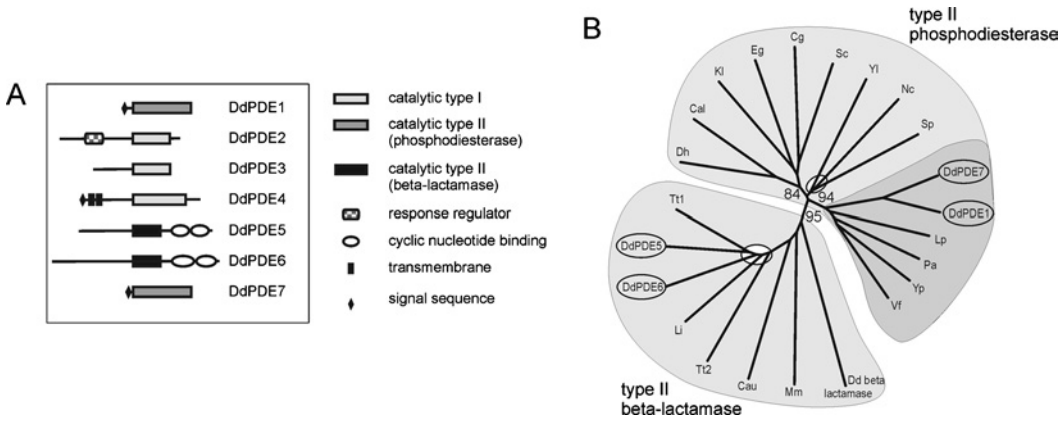

Figure 1 The PDE family of *D. discoideum*

(**A**) Seven PDEs have been recognized and identified in the completed genome of *D. discoideum*. The enzymes belong to the class I enzymes prevalent in higher eukaryotes, or to the class II enzymes that are present in lower eukaryotes and some bacteria. Based on bootstrap data of phylogenetic cluster analysis of type II enzymes, the class II enzymes are recognized as catalytic PDEs or catalytic metallo-β-lactamases. The localization and activity of the seven enzymes are regulated by signal sequences, transmembrane segments, a response regulator domain or cNB domains. (**B**) Cluster analysis of the catalytic domains of class II enzymes from prokaryotes and lower eukaryotes. All nodes have bootstrap values above 70 %, except for the nodes indicated by the small white ovals. Bootstrap values of the inner nodes are indicated. These bootstrap values and the length of the catalytic domain suggest two main groups, metallo-β-lactamases and PDEs; the latter may consist of two subgroups. The species abbreviations, gene identifiers and taxonomy are: Dh, *Debaryomyces hansenii*, gi|50424793, Eukaryota, Fungi; Cal, *Candida albicans*, gi|7548341, Eukaryota, Fungi; Kl, *Kluyveromyces lactis*, gi|50307193, Eukaryota, Fungi; Eg, *Eremothecium gossypii*, gi|44984787, Eukaryota, Fungi; Cg, *Candida glabrata*, gi|49526440, Eukaryota, Fungi; Sc, *Saccharomyces cerevisiae*, gi|6321189, Eukaryota, Fungi; Yl, *Yarrowia lipolytica*, gi|50554561, Eukaryota, Fungi; Nc, *Neurospora crassa*, gi|85086599, Eukaryota, Fungi; Sp, *Schizosaccharomyces pombe*, gi|3581909, Eukaryota, Fungi; Dd, *D. discoideum*; DdPDE1, gi|84080, DdPDE5, gi|21069535, DdPDE6, gi|66819225, DdPDE7, gi|66805301, Eukaryota, Mycetozoa; Lp, *Legionella pneumophila*, gi|53754670, Bacteria, Proteobacteria; Pa, *Pseudoalteromonas atlantica*, gi|76793857, Bacteria, Proteobacteria; Yp, *Yersinia pseudotuberculosis*, gi|77629947, Bacteria, Proteobacteria; Vf, *Vibrio fischeri*, gi|59711863, Bacteria, Proteobacteria; Ddβ-lactamase, *D. discoideum*, gi|66802803, Eukaryota, Mycetozoa; Mm, *Magnetospirillum magnetotacticum*, gi|23012958, Bacteria, Proteobacteria; Cau, *Chloroflexus aurantiacus*, gi |76165595, Bacteria, Chloroflexi; Tt, *Tetrahymena thermophila*; Tt1, gi|89287508, Tt2, gi|89296516, Eukaryota, Alveolata; Li, *Leptospira interrogans*, gi|45657830, Bacteria, Spirochaetes.

defective in multicellular development [28]. DdPDE5 (GbpA or PDED) is characterized as a cGMP-stimulated cGMP-specific class II PDE. The *ddpde5*$^-$ null cell strain exhibits a phenotype with strongly elevated levels of cGMP, both basal levels and especially after stimulation with cAMP. The biochemical phenotype of this *ddpde5*$^-$ null cell strain is similar to that observed in the mutant NP368, a Streamer F cell strain [6,29,30]. Finally, DdPDE6 (GbpB or PDEE) is a dual-specificity class II PDE that predominantly degrades intracellular cAMP and to a minor extent cGMP [6,30–32].

As mentioned above, *D. discoideum* cells possess two cyclic nucleotide PDEs on their cell surface, the cAMP/cGMP non-specific DdPDE1 and the cAMP-specific DdPDE4. In an attempt to characterize DdPDE4 we used the UK7 cell line which has a deletion of the gene encoding DdPDE1. As reported, UK7 cells have very low cell surface PDE activity [13], but surprisingly we observed that the majority of this residual activity is not DdPDE4, but resembles DdPDE1 in respect to catalytic properties and sensitivity to inhibitors. Upon completion of the *D. discoideum* genome-sequencing project, we recognized the presence of a new PDE, that we named DdPDE7 for its order of identification. In the present study we demonstrate that DdPDE7 is a close homologue of DdPDE1 in respect to amino acid sequence and enzymatic properties, and is responsible for the residual activity in UK7 cells.

To understand the function of PDEs in the regulation of intracellular and extracellular cAMP and cGMP levels it is essential to be confident that all PDEs in *D. discoideum* have been identified and characterized. Extensive searches of the now completed *D. discoideum* genome for class I or class II PDEs do not provide evidence for additional cyclic nucleotide PDEs. Searches for homologues of bacterial type III PDEs [33] have not yielded any potential candidate for this type of PDE in *D. discoideum*, suggesting that most probablly all cyclic nucleotide PDEs have now been characterized in *D. discoideum*. In the present study, we have used the kinetic properties of all enzymes to determine the contribution of each enzyme to the degradation of the three pools of cyclic nucleotides.

EXPERIMENTAL PROCEDURES

Construction of the overexpressor

To express the full length protein, a DNA fragment was PCR-amplified using the primers PDE7F: 5′-GAAGATCTAAAA-TGAAATATTTAATTTTAATTTTAATATTTTTTATTG-3′ and PDE7R: 5′-GAACTAGTTAAACAAATTGGTACACCTTGGT-TTG-3′. The BglII/SpeI fragment was cloned into the BglII-SpeI site of a modified MB74–GFP (green fluorescent protein) vector that contained a hygromycin- instead of a neomycin-resistance cassette [34], resulting in the plasmid pDM115-PDE7–GFP. Finally UK7 *D. discoideum* cells [13] were transfected with 5 μg of plasmid. Hygromycin-resistant cells were analysed; we will refer to this overexpressor as DdPDE7^{OE}.

Cluster analysis

Multiple sequence alignments were constructed using the CLUSTAL W program [35], followed by manual optimization. Distance matrices were constructed from the alignments with the PROTDIST program of the PHYLIP package, which uses the Dayhoff PAM 001 matrix for the calculation of evolutionary distances (Phylip 3.5) [36]. Phylogenetic trees were generated using the FITCH program of the PHYLIP package, with 1000 bootstrap replications to assess the reliability of the nodes.

Culture and incubation conditions

AX3, UK7, UK7/*regA*$^-$ and DdPDE7^{OE} strains were grown on HG5 medium (14.3 g/l Oxoid Pepton, 7.15 g/l yeast extract, 1.36 g/l $Na_2HPO_4 \cdot 12H_2O$, 0.49 g/l KH_2PO_4 and 10.0 g/l glucose)

Table 1 Three cyclic nucleotide pools regulated by seven PDEs

The intrinsic kinetic properties K_m, K_a and Hill coefficient were obtained from the present study and published data, DdPDE1 [41]; DdPDE2 [37] and our own measurements; DdPDE3 [27]; DdPDE4 [28]; DdPDE5 [6,50]; DdPDE6 [6,31]; DdPDE7, the present study. The V_{max} was determined using enzyme-specific assays as indicated in Table 2.

			Kinetic properties				
						V_{max} (pmol/min per 10^7 cells)	
Cyclic nucleotide pool	PDE	Class	K_m (μM)	Hill coeff.	K_a (μM)	Aggr.	Slugs
Extracellular cAMP	PDE1	II	0.75	0.8	–	95	115
	PDE7	II	12.5	0.79	–	22	11
	PDE4	I	9.6	1.0	–	13.45	296
Intracellular cAMP	PDE2	I	5	1.0	–	50	n.d.
	PDE6*	II	200/400	–	0.7	5200	n.d.
Intracellular cGMP	PDE3	I	0.2	1.0		2	n.d.
	PDE5*	II	5/20	–	0.16	400	n.d.
	PDE6*	II	800/1600	–	2.3	2400	n.d.

*These enzymes are activated by their substrate. In PDE5 this is shown to be mediated by reduction of the K_m, whereas the V_{max} remains the same; for DdPDE6 we assume the same kinetic mechanism. For each enzyme, two K_m values are presented, of the high-affinity and low-affinity form respectively. n.d., not determined.

either on a 9 cm dish or in shaking culture at 22 °C. The overexpressor DdPDE7^{OE} strain was grown in HG5 medium in the presence of 10 μg/ml hygromicin B. UK7/*regA*$^-$ cells were kindly provided by David Trayner and Rob Kay (MRC Laboratory of Molecular Biology, Cambridge, U.K.); the strain (HM1020) was made by disruption of the *regA* gene in UK7 cells using the vector as described for generating *regA*$^-$ cells [37].

PDE assays

D. discoideum cells were starved in 10 mM KH_2PO_4/Na_2HPO_4 (pH 6.5) for 5 h at a density of 10^7 cells/ml. Cells were centrifuged for 3 min at 300 ***g***, and the supernatant was saved as medium. The cells were washed with PDE lysis buffer [20 mM Hepes/NaOH (pH 7.0) and 1 mM EGTA] and resuspended in PDE lysis buffer at a density of 10^8 cells/ml. The PDE assay mixture (final concentrations) contained 20 mM Hepes/NaOH (pH 7.0), 1 mM EGTA, 5 mM $MgCl_2$, 10 nM [^{3}H]cAMP or 10 nM [^{3}H]cGMP as substrate, and 30 μl of cells or extracellular medium in a total volume of 100 μl. The assay mixture was incubated at room temperature (22 °C) for 30 min and the reaction was terminated by boiling for 1 min. The product was dephosphorylated by calf intestine phosphatase {1 unit of enzyme in 50 μl of calf intestine phosphatase buffer [50 mM Tris/HCl (pH 9.0), 0.1 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 1 mM spermidine] incubated for 1 h at 37 °C}. Finally, 150 μl of Dowex AG1 × 2 was added to remove the remaining substrate. After 10 min of incubation under regular mixing, samples were centrifuged for 2 min at 14000 ***g***, and the radioactivity in 200 μl of the supernatant was measured.

Calculation of degradation of three cyclic nucleotide pools

Using the measured kinetic constants of cAMP and cGMP degradation by the seven *D. discoideum* enzymes it is possible to calculate the contribution of each enzyme to the degradation of extracellular cAMP (by DdPDE1, DdPDE4 and DdPDE7), intracellular cAMP (by DdPDE2 and DdPDE6) and intracellular cGMP (by DdPDE3, DdPDE5 and DdPDE6). The equations of the enzymes with enzymatic constants of Table 1 are:

The allosteric enzymes PDE1and PDE7 (eqn 1):

$$v = V_{max}\frac{S^h}{K_{0.5}^h + S^h} \quad (1)$$

The Michaelis–Menten enzymes PDE2, PDE3 and PDE4 (eqn 2):

$$v = V_{max}\frac{S}{K_m + S} \quad (2)$$

The substrate-activated enzymes PDE5 and PDE6 (eqn 3):

$$v = V_{max}\left[\frac{S}{K_a + S}\frac{S}{K_m^H + S} + \frac{K_a}{K_a + S}\frac{S}{K_m^L + S}\right] \quad (3)$$

In these equations V_{max} is the maximal velocity of the enzyme, S is the substrate concentration, K_m is the Michaelis–Menten constant, $K_{0.5}$ is the apparent Michaelis–Menten constant, h is the Hill coefficient, K_a is the activation constant and K_m^L and K_m^H are the Michaelis–Menten constants of the basal and activated enzyme respectively.

The half-life of the substrate was calculated with eqn 4:

$$t_{0.5} = 0.6\frac{S}{v}\alpha \quad (4)$$

where $t_{0.5}$ is the half-life of substrate in min, S is the substrate concentration in μM, v is the calculated rate of degradation in pmol/min per 10^7 cells, and α is the volume of 10^7 cells in μl. The proportionality constant 0.6 is the average between ln2 0.69 (for first-order degradation at $S \ll K_m$) and 0.5 (for zero-order degradation at $S \gg K_m$). The cell density during aggregation is 10^8 cells/ml yielding $\alpha = 100$ μl for cell surface enzymes during aggregation. The volume of a cell is about 630 μm^3 [38] yielding $\alpha = 6.3$ μl for cytosolic enzymes. We assume that in slugs the volume between cells is in the same order as the volume of the smaller cells ($\alpha = 5$ μl).

RESULTS

Deduced amino acid sequence of DdPDE7

The deduced DdPDE7 protein consists of 425 amino acids, of which the first 17 amino acids are predicted to form a signal peptide with probable cleavage between amino acids 17 and 18 (Figures 1A and 2). The predicted catalytic domain starts at amino acid 22 and continues until the C-terminus and belongs to the group of class II PDEs that are present in lower eukaryotes and bacteria, but not in higher eukaryotes. The homologous DdPDE1 is somewhat larger (452 amino acids), mainly at the N-terminus. The deduced amino acid sequence of DdPDE1 is also predicted to

Figure 2 Alignment of DdPDE1 and DdPDE7

The deduced amino acid sequence of DdPDE1 and DdPDE7. Underlined are the predicted signal sequences with cleavage between amino acid 17 and 18 for DdPDE1 and 23 and 24 for DdPDE7. A potential hydrophobic transmembrane segment is indicated in small italic letters. The asterisk indicates a potential GPI anchor.

contain a signal sequence with a probable cleavage site between amino acids 23 and 24, and a catalytic domain that starts at amino acid 52. The catalytic domains of DdPDE1 and DdPDE7 share 62 % amino acid identity and 78 % identical or conserved amino acids (Figure 2).

To further characterize the homology between DdPDE1 and DdPDE7, we investigated the genomic organization of the respective genes [39] at http://dictybase.org/. DdPDE7 is encoded by BC5V2_0_00956 on chromosome 5, whereas DdPDE1 is encoded by the *pdsA* gene on chromosome 4. DdPDE7 lies two genes downstream of DdPDE4 in the opposite orientation. DdPDE7 has no introns in the coding region, whereas DdPDE1 has one intron in the coding region and two introns in the non-coding region. The upstream region of DdPDE7 containing the putative promoter region does not show a clear resemblance to the complex promoter region of DdPDE1 (results not shown).

Homology and cluster analysis of the catalytic domain

DdPDE7 belongs to the group of class II cyclic nucleotide PDEs, which are present in some bacteria and lower eukaryotes. DdPDE1 also belongs to this class, as well as DdPDE5 and DdPDE6, although the catalytic domains of DdPDE5 and DdPDE6 are shorter and resemble metallo-β-lactamases. We made a multiple sequence alignment of the catalytic domains of these four *D. discoideum* PDEs together with class II PDEs from other lower eukaryotes and bacteria, and performed a phylogenetic/protein cluster analysis (Figure 1B). The result demonstrates at least two groups of class II cyclic nucleotide PDEs (bootstrap 95 %): the originally defined type II PDEs [40], and a group resembling metallo-β-lactamases. Based on a bootstrap value of 94 %, the type II PDEs may consist of two subgroups, one subgroup with DdPDE1, DdPDE7 and presently four enzymes from a few prokaryotes (all belonging to the class of γ-proteobacteria), and a subgroup of about ten enzymes from eukaryotes (all belonging to the division Ascomycota of the Fungi kingdom). Interestingly, the proteins of the metallo-β-lactamase type II PDEs all harbour a metallo-β-lactamase domain and one or usually two cNB (cyclic nucleotide-binding) domains. Sequences are found in a few prokaryotes, and in the eukaryotes *Tetrahymena thermophila* and *D. discoideum*. For *D. discoideum* DdPDE5 and DdPDE6, it has been demonstrated that the metallo-β-lactamase domain possesses PDE activity, which is activated by cAMP or cGMP binding to the cNB domains [31].

Catalytic activity of DdPDE7

Previously, only two PDEs in *D. discoideum* were known that have their catalytic activity exposed on the cell surface, DdPDE1 and DdPDE4 [28]. With the recognition of DdPDE7 as a close homologue of DdPDE1, we have carefully analysed our older data on the residual PDE activity of the *ddpde1*-null cell line UK7. DdPDE1 is a dual cAMP/cGMP PDE that is strongly inhibited by DTT (dithiothreitol) and insensitive to IBMX (3-isobutyl-1-methylxanthine), whereas DdPDE4 is insensitive to DTT but inhibited by IBMX.

Wild-type cells that have been starved for 5 h have a very active PDE activity on their surface (3000 fmol/min per 10^7 cells at 10 nM cAMP), which is essentially completely inhibited by 10 mM DTT and insensitive to IBMX (Figure 3A). UK7 cells have a 50-fold reduction of surface PDE activity (60 fmol/min per 10^7 cells), implying that DdPDE1 contributes by far the majority of PDE activity on the surface of *D. discoideum* cells that have been starved for 5 h, as has been discussed previously in [13]. The residual activity on UK7 cells is inhibited only slightly by IBMX; the 15 % inhibition (9 fmol/min per 10^7 cells) most likely represents the IBMX-sensitive DdPDE4 that is known to be expressed at these low levels in cells that have been starved for 5 h [28]. The residual activity of UK7 cells is inhibited 90 % by DTT, suggesting that UK7 cells may express a homologue of DdPDE1.

To demonstrate that the DTT-sensitive activity of UK7 cells is attributed to DdPDE7, we overexpressed the *ddpde7* gene in UK7 cells (Figure 3B). The DTT-sensitive PDE activity of UK7 cells is present approx. 15 % at the cell surface and approx. 85 % secreted into the medium. Overexpression of DdPDE7 leads to a 2.4-fold increase of PDE activity, both in the medium and expressed on the

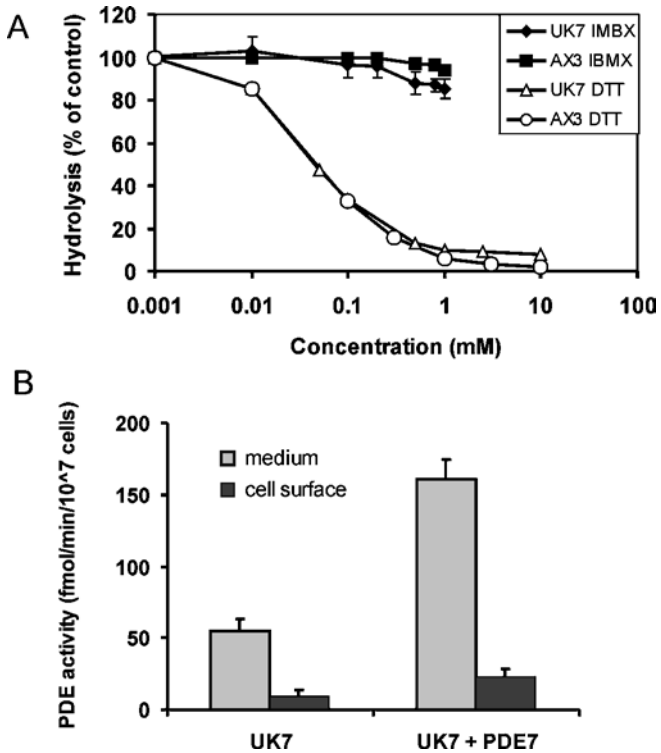

Figure 3 Initial characterization of DdPDE7

(**A**) Inhibition by IBMX and DTT of PDE activity at the surface of wild-type AX3 cells and UK7 cells with a deletion of DdPDE1 that had been starved for 5 h. The activity in the absence of inhibitors is set at 100 % (2950 fmol/min per 10^7 cells in AX3 and 61 fmol/min per 10^7 cells in UK7). The results show that the residual PDE activity exposed on UK7 cells is inhibited strongly by DTT and only partly by IBMX. (**B**) Over-expression of DdPDE7 in UK7 cells reveal enhanced activity both on the cell surface and secreted in the medium.

surface at the same ratio as in control cells. The enhanced activity of UK7/DdPDE7^{OE} cells hydrolyses both cAMP and cGMP and is sensitive to inhibition by DTT and not by IBMX (results not shown). These properties of UK7/DdPDE7^{OE} cells are essentially identical to those of UK7 cells. We conclude that DdPDE7 is the major PDE activity of UK7 cells that have been starved for 5 h.

Kinetics of DdPDE7

PDE activity was measured with different concentrations of cAMP or cGMP using intact UK7 cells. The Eady–Hofstee plot (Figure 4) reveals a slight negative co-operativity as has been observed for DdPDE1 (see [41] for the kinetic data of DdPDE1). The inset shows a Hill plot, yielding a Hill coefficient of 0.79 for DdPDE7, compared with 0.8 for DdPDE1 [41]. At subsaturating concentrations of 10 nM, UK7 cells hydrolyse cAMP about 1.6-fold faster than cGMP, indicating that DdPDE7 is a dual-specificity enzyme, as has also been observed for DdPDE1. However, the affinity of DdPDE7 for cAMP is much lower than that of DdPDE1 (the apparent K_m is 0.8 μM cAMP for DdPDE1 and 12.5 μM for DdPDE7). Both DdPDE1 and DdPDE7 have a 2- to 3-fold lower affinity for cGMP compared with cAMP. In contrast, DdPDE7 appears to have a 2.6-fold higher V_{max} for cGMP compared with cAMP, while DdPDE1 has a 1.5-fold lower V_{max} for cGMP than for cAMP. Thus both DdPDE1 and DdPDE7 are dual cAMP/cGMP specificity PDEs with negative co-operativity. However, the kinetic constants are very different for DdPDE1 and DdPDE7, especially the 15-fold lower cAMP affinity of DdPDE7 compared with DdPDE1.

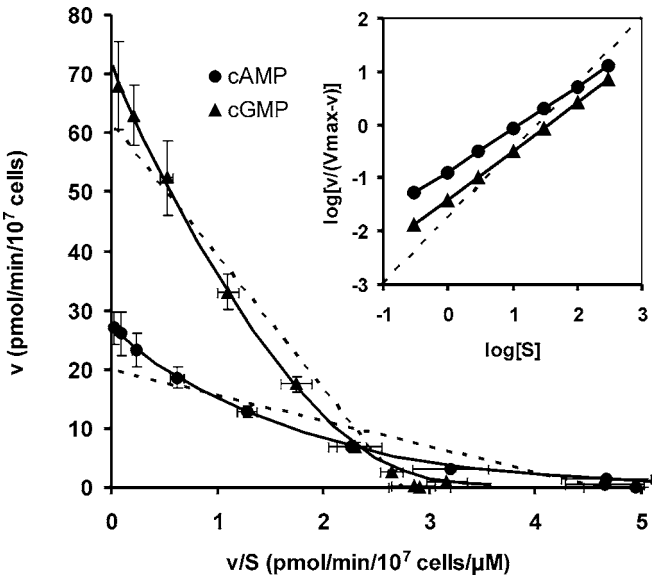

Figure 4 Kinetics of DdPDE7

UK7 cells that had been starved for 5 h were incubated with different concentrations of cAMP or cGMP. The hydrolysis was measured after 30 min and expressed as an Eady–Hofstee plot in the main figure and as a Hill plot in the inset. The dotted lines were fitted for Michaelis–Menten kinetics using linear regression, while the straight lines were fitted for negative co-operativity using the Hill equation. The deduced kinetic constants for the Hill equation are, for cAMP apparent $K_m = 12.5$ μm, $V_{max} = 28$ pmol/min per 10^7cells, Hill coefficient = 0.79, and for cGMP, apparent $K_m = 36$ μm, $V_{max} = 72$ pmol/min per 10^7cells and Hill coefficient = 0.9.

DdPDE1, DdPDE4 and DdPDE7 activity during development

Extracellular cAMP is degraded by the IBMX-sensitive DdPDE4 and DTT-sensitive DdPDE1 and DdPDE7. We have previously determined total cell surface cAMP–PDE activity during development, and the proportion of this activity that is inhibited by IBMX and DTT [28]. The IBMX-sensitive activity is attributed to DdPDE4, whereas the DTT-sensitive activity is caused by DdPDE1 and DdPDE7. Since the latter two enzymes have nearly identical properties it is difficult to determine the activities of DdPDE1 and DdPDE7 during development of wild-type cells. We used UK7/*regA*$^-$ cells that have deletions in both DdPDE1 and DdPDE2/RegA; due to the deletion of DdPDE1, all DTT-sensitive activity is due to DdPDE7, whereas deletion of DdPDE2 allows the aggregation-defective UK7 cell to develop up to the stage of tight aggregates with a tip. We measured the hydrolysis of [^{3}H]cAMP by UK7/*regA*$^-$ cells in the absence and presence of 10 mM DTT to discriminate between the DTT-sensitive DdPDE1 and the DTT-insensitive DdPDE4. The results show a steady increase of cell surface PDE activity during development. During the first 5 h of development nearly all PDE activity is DTT sensitive (i.e. attributed to DdPDE7). At later stages of development, this DTT-sensitive activity declines (Figure 5A) and most of the PDE activity becomes sensitive to IBMX ([28] and results not shown). These results suggest that vegetative cells have a low activity of DdPDE7, which increases about 3-fold during cell aggregation, and declines in the multicellular stage. In Figure 5(B) we calculated the activity of the three enzymes by combining the present data on the activity of DdPDE7 in UK7/*regA*$^-$ cells with previously determined DdPDE1 + DdPDE7 and DdPDE4 activity in wild-type cells, on the assumption that wild-type and UK7/*regA*$^-$ cells express the same amount of DdPDE7 activity. DdPDE1 represents by far the most prominent activity during development at a substrate concentration of 10 nM. Maximal DdPDE7 activity is about 75 fmol/min per 10^7 cells during cell

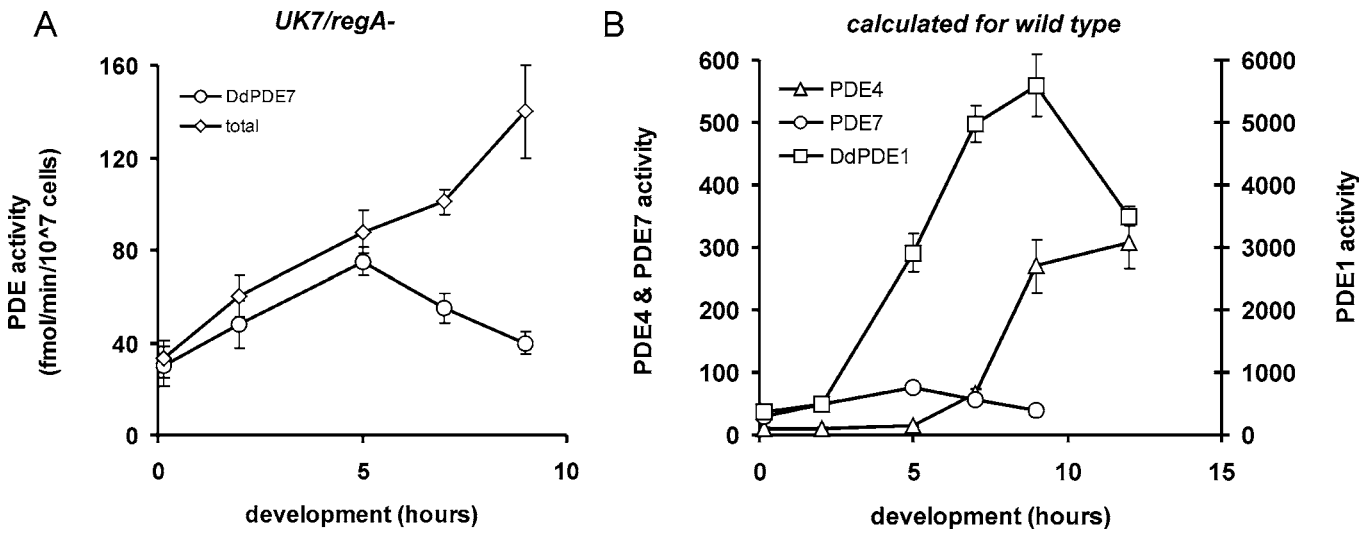

Figure 5 DdPDE7 activity during development

(**A**), UK7/*regA*$^-$ cells were starved on plates for the times indicated, harvested, washed and assayed for cell surface cAMP hydrolysis using 10 nM [^{3}H]cAMP. Cell aggregation was observed at 6 h of development, and tight aggregates and slugs were observed at 9 h; development was arrested at this stage. (**B**) Activity of DdPDE1, DdPDE4 and DdPDE7 during development, deduced from (**A**) for DdPDE7, and from Figure 6 in Bader et al. [28] for DdPDE1 and DdPDE4.

Table 2 Enzyme-specific assays for seven PDEs in *D. discoideum* cells

				Specific assay	
Enzyme	Location	Inhibitor	Substrate	enzyme preparation	substrate condition
DdPDE1	Cell surface	DTT	cAMP cGMP	Intact cells with IBMX	Activity with cAMP minus DdPDE7
DdPDE2	Cytosol	IBMX	cAMP	Cytosol with DTT	Reduction activity with cAMP by IBMX*
DdPDE3	Cytosol	IBMX	cGMP	Cytosol with DTT	Reduction activity with cGMP by IBMX*
DdPDE4	Cell surface	IBMX	cAMP	Intact cells with DTT	Reduction activity with cAMP by IBMX*
DdPDE5	Cytosol	–	cGMP	Cytosol with DTT and IBMX	Activity with cGMP
DdPDE6	Cytosol	–	cAMP cGMP	Cytosol with DTT and IBMX	Activity with cAMP
DdPDE7	Cytosol	DTT	cAMP cGMP	Intact *Ddpde1*$^-$*/Ddpde2*$^-$ cells (UK7/regA$^-$) with IBMX	Activity with cAMP
DdPDE1 + DdPDE7	Cell surface	DTT	cAMP cGMP	Intact cells with IBMX	Activity with cAMP

*The activity is measured in the absence and presence of IBMX; the difference of these activities is attributed to the enzyme indicated. The concentrations of inhibitors used were 0.8 mM IBMX or 5 mM DTT.

aggregation, compared with about 3000 fmol/min per 10^7 cells for DdPDE1, both measured at 10 nM cAMP.

Contribution of seven PDEs in the degradation of cAMP and cGMP pools

In *D. discoideum* three cyclic nucleotide pools are pivotal for the organism. Extracellular cAMP induces chemotaxis and differentiation, which are mediated by intracellular cGMP and cAMP respectively. Since all *D. discoideum* PDEs are now characterized we can calculate the contribution of each enzyme to the degradation of these three important pools of cyclic nucleotides. We assume that at different developmental stages each enzyme is expressed at different levels, but that the intrinsic kinetic properties do not change. This assumption implies that the sensitivity to inhibitors, substrate specificity, K_m, K_a and Hill coefficient are constant, whereas the V_{max} is different depending on the developmental stages. First, we obtained the intrinsic kinetic constants K_m, K_a and Hill coefficient of all enzymes from present and published data (see Table 1 for data and references). Secondly, we used the location of the enzyme, the substrate specificity and the sensitivity to inhibitors to derive specific assay conditions to determine the V_{max} for each enzyme (Table 2). These experiments were performed in wild-type cells, except for DdPDE7, for which we have to rely on the measurements in UK7/*regA*$^-$ null cells, because in wild-type cells we cannot discriminate between DdPDE1 and DdPDE7. Subsequently, we used the observed values of K_m, V_{max} and co-operativity (Table 1) to calculate the activity of each enzyme at different substrate concentrations (Figure 6). In this analysis we also calculated the half-life of cAMP or cGMP degradation at different substrate concentrations. To evaluate the outcome of these calculations, we used published data to estimate the cyclic nucleotide concentration of these three pools *in vivo* (indicated by the arrows in Figure 6).

Extracellular cAMP is degraded by DdPDE1, DdPDE4 and DdPDE7. During cell aggregation, extracellular cAMP waves are propagated every 5 min with concentrations that oscillate between approx. 1 nM and approx. 1 μM [42]. The half-life of 10 nM

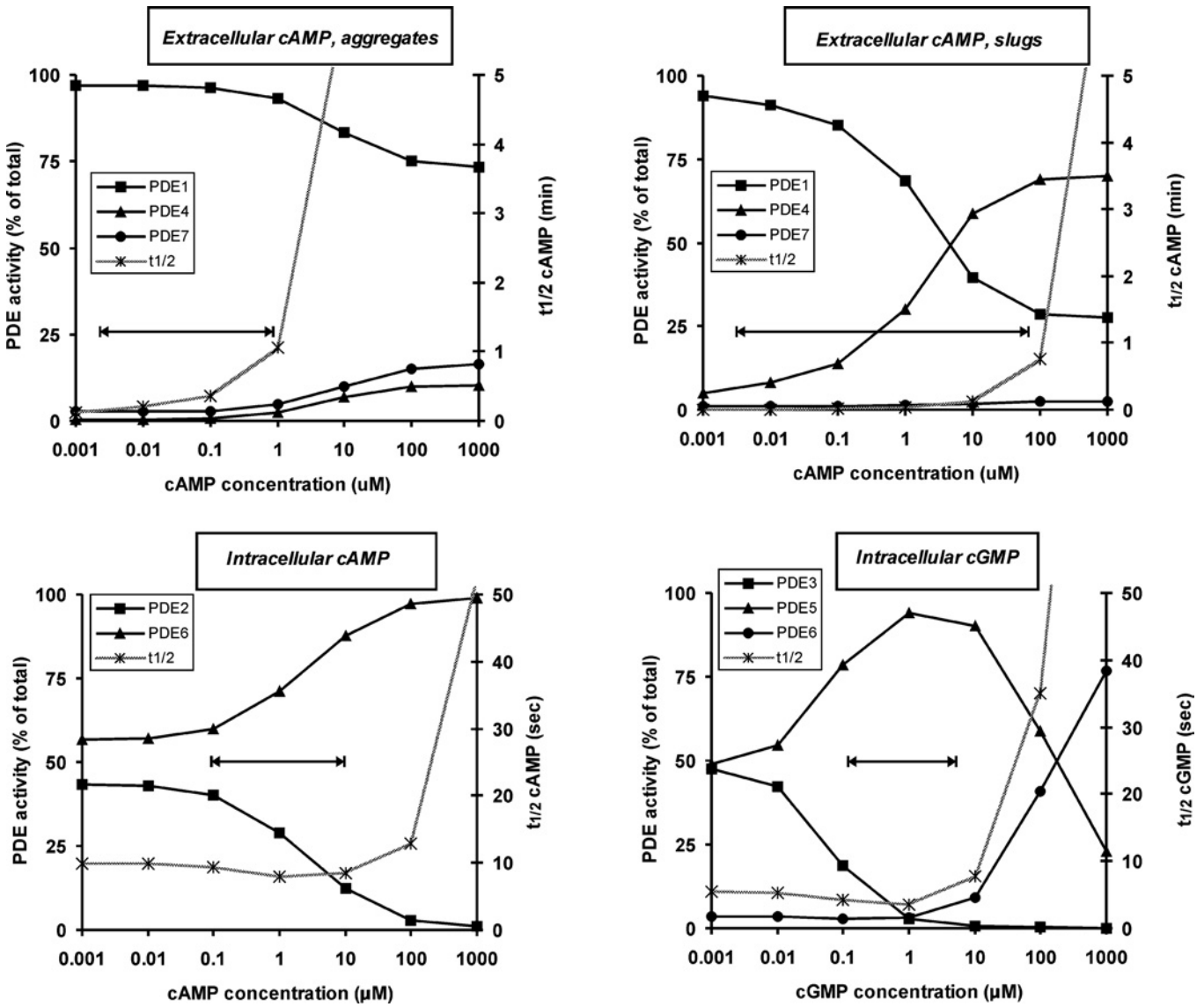

Figure 6 Contribution of the seven PDEs to the hydrolysis of three cyclic nucleotide pools

(**A**) Extracellular cAMP during aggregation; (**B**) extracellular cAMP in slugs; (**C**) intracellular cAMP during aggregation; (**D**) intracellular cGMP during aggregation. The activity of each enzyme was calculated at different substrate concentrations using the equations presented in the Experimental procedures section and the kinetic constants as presented in Table 1. From these activities we calculated the half-life of degradation of the indicated substrate concentration, and the relative contribution of the participating enzymes in the degradation of substrate. The arrows indicate the range of substrate concentrations that have been measured *in vivo* during cell stimulation.

cAMP in a suspension of 10^8 cells/ml is calculated to be about 10 s, as was observed experimentally [43]. The degradation of 10 nM cAMP is mediated largely by DdPDE1 (approx. 97 %), and only slightly by DdPDE4 (approx. 0.3 %) and DdPDE7 (approx. 2.6 %). At substrate concentrations above 1 μM the half-life of cAMP increases sharply because the activity of DdPDE1 with its K_m of 0.8 μM becomes saturated. The degradation of 1 μM cAMP is still mediated predominantly by DdPDE1 (approx. 93 %), and to some extent by DdPDE4 (approx. 2 %) and DdPDE7 (approx. 5 %).

In the multicellular stage, the half-life of cAMP is calculated to be much shorter than during cell aggregation because the cell density is much higher and the PDE activity per cell is only slightly lower than during aggregation. Degradation of low cAMP concentrations is again mainly mediated by DdPDE1 (approx. 94 %), while DdPDE4 (approx. 5 %) and DdPDE7 (approx. 1 %) make less of a contribution. Due to the small intercellular space, the cAMP concentration in slugs may easily reach a concentration of 10 or 100 μM [29,44,45]. At these high cAMP concentrations DdPDE1 is completely saturated and degradation also depends on the enzymes with a lower affinity for cAMP, such as DdPDE4 and DdPDE7. Since DdPDE4, but not DdPDE7, is expressed at relatively high levels in slugs compared with single cells, DdPDE4 has a significant contribution of approx. 58 % in the degradation of 10 μM cAMP in slugs. DdPDE7 contributes approx. 2 % only and DdPDE1 the remaining 40 %.

Intracellular cAMP is a second messenger that transduces the extracellular cAMP signal to induce development. After stimulation, intracellular cAMP may reach a concentration of 5 μM (25 pmol/10^7 cells [46,47]). Intracellular cAMP is degraded by the high-affinity DdPDE2 and the low-affinity DdPDE6. At low cAMP concentrations the half-life of cAMP is approx. 15 s, and

cAMP is degraded by both DdPDE2 (approx. 40 %) and DdPDE6 (approx. 60 %). At a cAMP concentration of 10 μM, which is just above the maximal intracellular cAMP concentration, the low-affinity DdPDE6 becomes more important and degrades about 85 % of cAMP. At this high cAMP concentration, the half-life of cAMP is still only 15 s, and even at 100 μM cAMP degradation is still very fast.

Intracellular cGMP mediates chemotaxis and is degraded by DdPDE3, the cGMP-stimulated DdPDE5 and possibly DdPDE6. After stimulation, intracellular cGMP may reach a concentration of 3 μM (15 pmol/10^7 cells; [43]). At a basal cGMP concentration of 0.03 μM, both the high-affinity DdPDE3 (approx. 40 %) and the low-affinity DdPDE5 (approx. 60 %) contribute in the degradation of cGMP. At elevated cGMP levels of 3 μM, DdPDE5 (approx. 90 %) becomes the predominant enzyme in the degradation of cGMP. At very high cGMP levels, which may occur after osmotic stimulation [48,49], DdPDE6 may become increasingly important in the degradation of intracellular cGMP.

DISCUSSION

The identification of all PDEs in *D. discoideum* is very important for understanding signal transduction and development in *D. discoideum*. The completed genome sequence [39] uncovers seven PDEs. In the present paper we have characterized the last member, DdPDE7. The deduced amino acid sequence and the kinetic properties reveal that DdPDE7 is highly homologous to DdPDE1. Enzyme activity measurements reveal that both DdPDE1 and DdPDE7 are secreted into the medium and are attached to the cell surface. The deduced amino acid sequence reveals that both contain a signal sequence that is probablly responsible for secretion. The mechanism for membrane association is not known for DdPDE1 (R. H. Kessin, personal communication) and DdPDE7. The hydrophobic signal sequence could function as a transmembrane segment, or the proteins may be attached to the membrane by potential GPI (glycosylphosphatidylinositol) anchors (Asn^{436} in DdPDE1 and Asn^{409} in PDE7). Both DdPDE1 and DdPDE7 are PDEs with dual cAMP/cGMP specificity, and are strongly inhibited by DTT and insensitive to IBMX. Although similar, the kinetic and inhibitory constants of DdPDE1 and DdPDE7 are significantly different. The most striking difference is the approx. 15-fold higher K_m and the approx. 5-fold lower V_{max} of DdPDE7 for cAMP compared with DdPDE1.

The family of *D. discoideum* PDEs can now be placed in perspective. Cyclic nucleotides are hydrolysed by two unrelated classes of PDEs, the class I enzymes that are present in most eukaryotes and prokaryotes, and the class II enzymes that are found only in some lower eukaryotes and prokaryotes. In *D. discoideum* three class I enzymes and four class II enzymes have been identified. The phylogeny of the *D. discoideum* class I enzymes has been described in detail showing that DdPDE4 resembles most closely mammalian PDE8, while DdPDE2 and DdPDE3 are not closely related to a specific mammalian PDE [28]. The four *D. discoideum* class II enzymes belong to two subgroups, type II metallo-β-lactamases and type II PDEs. DdPDE5 and DdPDE6 have catalytic domains resembling metallo-β-lactamase. In addition, these enzymes possess two cNB domains that upon binding of cGMP or cAMP activate the catalytic domain [6,31,50]. We identified about ten putative PDEs that have a similar topology with a metallo-β-lactamase and usually two cNB domains; five sequences were identified in the eukaryote *Tetrahymena thermophila*, and one enzyme each in a few prokaryotes. The sequences are generally annotated as proteins with cNB domains, the metallo-β-lactamase is often not recognized, and none is recognized as a potential PDE. It will be interesting to investigate whether these proteins are cyclic nucleotide-regulated PDEs. It is interesting to note that the *D. discoideum* DdPDE5 is a cGMP-stimulated cGMP-specific PDE with kinetic constants and cGMP-specificity very similar to the mammalian PDE5, despite the completely different evolutionary origin of these enzymes. DdPDE5 has a type II metallo-β-lactamase catalytic domain that is activated by cNB domains, whereas mammalian PDE5 has a class I catalytic domain that is activated by GAF domains. The other two *D. discoideum* class II PDEs, DdPDE1 and DdPDE7, belong to the group of type II PDEs, which have a significantly larger catalytic domain than β-lactamases. DdPDE1 and DdPDE7 belong to a small subgroup together with four sequences from proteobacteria, while the other subgroup harbours sequences from only eukaryotes.

D. discoideum cells contain three pools of cyclic nucleotides with different functions: extracellular cAMP, intracellular cAMP and intracellular cGMP (Table 1). It is striking that each cyclic nucleotide pool is regulated by a combination of class I and class II PDEs, and that the enzymes in each pool have different kinetic properties: usually, one enzyme has a high affinity for the substrate and a relatively low capacity, while the other enzyme has a lower affinity and higher capacity. These properties allow the degradation of the substrate over a wide range of concentrations to occur with approximately first-order kinetics. Since all *D. discoideum* PDEs are now characterized, we have estimated the contribution of each enzyme in the degradation of the three important pools of cyclic nucleotides during development. These three cyclic nucleotide pools fluctuate in their concentrations (Figure 6). Interestingly, the calculated half-life of cyclic nucleotides is still within a functional range at the highest concentrations that are expected to occur *in vivo*, but will increase dramatically at higher concentrations. Two examples may illustrate this point. First, intracellular cGMP may reach concentrations between 0.01 and 2 μM. The combination of the high-affinity low-capacity DdPDE3 and low-affinity high-capacity DdPDE5 results in a half-life that remains nearly constant between 0.01 and 2 μM cGMP, but increases strongly above 10 μM cGMP. Secondly, extracellular cAMP oscillates with a periodicity of approximately 5 min, which dictates that extracellular cAMP must be degraded with a half-life of approx. 1 min or less. During cell aggregation cAMP levels do not increase beyond a concentration of 1 μM, which can be effectively degraded by the high-affinity DdPDE1. In slugs, however, extracellular cAMP levels may easily reach a concentration of 10 or 100 μM, due to the small space between the cells of a slug. With the increased expression of the low-affinity DdPDE4, this high concentration is effectively degraded by which the half-life of 100 μM cAMP remains below 1 min. Thus it appears that the combination of enzymes that degrades each pool is tuned in such a way that the concentration and lifetime of the substrate remain under control.

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